JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 2009, p. 988–993 Vol. 47, No. 4
0095-1137/09/$08.00+0 doi:10.1128/JCM.01346-08

Universal Detection and Identification of Avian Influenza Virus by Use of Resequencing Microarrays▽†

Baochuan Lin,[1]* Anthony P. Malanoski,[1] Zheng Wang,[1] Kate M. Blaney,[2] Nina C. Long,[2] Carolyn E. Meador,[2] David Metzgar,[3] Christopher A. Myers,[3] Samuel L. Yingst,[4] Marshall R. Monteville,[4] Magdi D. Saad,[4] Joel M. Schnur,[1]‡ Clark Tibbetts,[5] and David A. Stenger[1]

Center for Bio/Molecular Science & Engineering, Code 6900, Naval Research Laboratory, Washington, D.C. 20375[1]; Nova Research, Incorporated, Alexandria, Virginia 22308[2]; Department of Respiratory Disease Research, Naval Health Research Center, San Diego, California 92106[3]; Naval Medical Research Unit 3, Cairo, Egypt[4]; and TessArae, LLC, Potomac Falls, Virginia 20165[5]

Received 15 July 2008/Returned for modification 9 January 2009/Accepted 17 February 2009

Zoonotic microbes have historically been, and continue to emerge as, threats to human health. The recent outbreaks of highly pathogenic avian influenza virus in bird populations and the appearance of some human infections have increased the concern of a possible new influenza pandemic, which highlights the need for broad-spectrum detection methods for rapidly identifying the spread or outbreak of all variants of avian influenza virus. In this study, we demonstrate that high-density resequencing pathogen microarrays (RPM) can be such a tool. The results from 37 influenza virus isolates show that the RPM platform is an effective means for detecting and subtyping influenza virus, while simultaneously providing sequence information for strain resolution, pathogenicity, and drug resistance without additional analysis. This study establishes that the RPM platform is a broad-spectrum pathogen detection and surveillance tool for monitoring the circulation of prevalent influenza viruses in the poultry industry and in wild birds or incidental exposures and infections in humans.

Recent outbreaks of Nipah virus, severe acute respiratory syndrome virus, and avian influenza virus reiterate the importance of zoonotic microbes as potential threats to human health (26). Influenza virus causes particular concern, owing to the repeated nature of influenza pandemics and their potential to result in significant mortality, exemplified by the 1918 influenza pandemic. To date, most influenza A virus subtypes (e.g., H2N2 and H10N7) resulting from combinations of the 16 hemagglutinin (*HA*) and 9 neuraminidase (*NA*) types are detected in wild birds and poultry (9, 40). In a survey of 38,609 samples from wild birds, 55 different *HA* and *NA* subtype combinations were identified, with H4N6 appearing as the most prevalent subtype, followed by H7N7 and H6N2 (24). The emergence of H5N1 since 1997 in Asia, the Middle East, Europe, and Africa amplifies concerns about the broad natural diversity of host species (mostly aquatic and migratory birds) which provide rapid geographical distribution of new strains and enable transmission to human populations (24, 25). Recent major outbreaks in domestic poultry and wildfowl populations caused by different serotypes, including H5N1, H5N2, H7N1, H7N3, H7N4, and H7N7, indicate that the threat is not from a single serotype (25).

Detection and discrimination of all potential influenza A virus subtypes is needed to identify the introduction of zoonotic strains to humans, monitor the status of these pathogens in their natural hosts, and minimize epidemic spread if transmissible human infections occur. An effective surveillance assay could rapidly detect and identify all subtypes of avian influenza virus and provide useful secondary information related to specific functional mutations which alter pathogenicity or drug resistance. For example, the low-pathogenicity H5N1 viruses should be differentiated from the highly pathogenic strains by a mutation in an *HA* cleavage site (a multibasic cleavage motif, PQRERRRKKRG), a deletion of 20 amino acids in the NA protein, and a signature amino acid substitution, E627K, in the PB2 protein (1, 23).

Viral culture paired with serological *HA* typing is the current standard method for detecting and typing influenza A viruses. These procedures are time-consuming, taking days or even weeks to provide specific results. Several molecular diagnostic approaches including reverse transcription (RT)-PCR, real-time PCR, PCR–enzyme-linked immunosorbent assay, and spotted oligonucleotide microarrays provide fast and sensitive alternatives to viral culture (5, 8, 12–15, 20, 23, 27, 31, 33, 34, 38). While promising, these methods either are limited to detecting only a few subtypes or provide a very limited range of genetic resolution. Additional time-consuming characterization, such as direct sequencing, is required for analysis of strain variations and specific mutations that contribute to or predict influenza virus pathogenicity, host range, drug resistance, and vaccine efficacy.

Alternative strategies, such as the use of RT-PCR coupled with pyrosequencing (7, 28), RT-PCR–electrospray ionization

* Corresponding author. Mailing address: Center for Bio/Molecular Science & Engineering, Code 6900, Naval Research Laboratory, 4555 Overlook Avenue, S.W., Washington, DC 20375. Phone: (202) 767-0289. Fax: (202) 767-9594. E-mail: baochuan.lin@nrl.navy.mil.

† Supplemental material for this article may be found at http://jcm.asm.org/.

‡ Current address: College of Science, George Mason University, Fairfax, VA 22030.

▽ Published ahead of print on 11 March 2009.

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
JAN 2009		**00-00-2009 to 00-00-2009**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Universal Detection and Identification of Avian Influenza Virus by Use of Resequencing Microarrays	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Naval Research Laboratory,Center for Bio/Molecular Science & Engineering, Code 6900,Washington,DC,20375**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**Same as Report (SAR)**	**6**	

(ESI)-mass spectrometry (MS) (30), or resequencing pathogen microarrays (RPM) (6, 16, 17, 19, 36), allow tracking of genetic changes and supply subspecies identification. The pyrosequencing technique is currently limited to short fragments and is applied to detection of H5N1 or selected drug resistance markers (3, 4, 7, 28). The RT-PCR–ESI-MS method, developed for detection of all avian serotypes, has, to date, only demonstrated tracking of genetic changes in human influenza virus samples. The RPM technology is the only one of these technologies currently under development for simultaneous detection and identification of influenza A virus variants together with a large number of other viral and bacterial pathogens that may elicit similar flulike illnesses. Furthermore, the RPM technology separates and partially decouples the amplification of limiting templates by multiplex RT-PCR from the selection of microarray contents and detection capability, which alleviates constraints on primer selection while still providing the required specificity.

Herein, we investigate the performance of new versions of the respiratory pathogen microarray (TessArray RPM-Flu 3.0 and 3.1, subsequently designated RPM-Flu (see Table S1 in the supplemental material) for detection and differential identification of all subtypes of the influenza A virus *HA* and *NA* genes in a single-pass assay. Previous studies demonstrated the ability of RPM technology to detect targeted pathogens with analytical and clinical sensitivities and specificities that are similar to (or improved over) those for existing technologies, while simultaneously providing sequence information for strain resolution (6, 16, 17, 19, 22, 36). The RPM-Flu arrays are designed and constructed to allow thorough coverage of 86 bacterial and viral agents, including respiratory pathogens and zoonotic organisms considered to be significant risks for human health, e.g., severe acute respiratory syndrome virus.

About 30% of the RPM-Flu array is dedicated to targeting all 16 *HA* and 9 *NA* alleles of avian influenza A viruses. The *HA* and *NA* genes represented on the microarray are based on prevalent strains of influenza A viruses circulating in the avian population within the last few years. Previous studies have shown that a single sequence on a resequencing microarray could reliably detect and serotype strains with as much as 10 to 15% variation (16, 18, 19, 36). Thus, this technology has the potential to detect all possible influenza A virus *HA* and *NA* subtype combinations. The data presented here demonstrate that the RPM platform is an effective means for universal detection and identification of all subtypes of avian influenza A viruses and provides useful secondary information related to pathogenicity and drug resistance.

(This work was presented in part at the 107th general meeting of the American Society for Microbiology, 2007.)

MATERIALS AND METHODS

Influenza A virus *HA* and *NA* templates. *HA*1, *HA*3, *NA*1, and *NA*2 sequences were amplified from total nucleic acids extracted from live, trivalent, nasally administered influenza vaccine (FluMist 2004/05; Medimmune, Inc., Gaithersburg, MD). The influenza A virus H5N1 (Asian lineage) real-time-PCR/RT-PCR-positive control (catalog no. VA2711; Centers for Disease Control and Prevention Laboratory Response Network) was kindly provided by the Centers for Disease Control and Prevention (Atlanta, GA). The influenza A virus *HA*2, *HA*4-16, and *NA*3-9 templates were synthesized by BlueHeron Biotechnology, Inc. (Bothell, WA) (see Table S2 in the supplemental material).

Specimen collection and processing. Cloacal swabs from migratory birds (mostly waterfowl), cloacal and/or tracheal swabs from commercial poultry, tracheal and lung tissue samples from dead birds, human throat swabs, and one human lung sample taken from a deceased patient were collected by Naval Medical Research Unit 3 (NAMRU3; Cairo, Egypt). These samples were cultured using chicken eggs and/or MDCK cells. Sample collection and viral culture techniques were performed as described in the World Health Organization Manual On Animal Influenza Diagnosis and Surveillance, Version 2002.5, revision 1 (39). Total RNA was extracted from culture isolates; 1:100 to 1:1,000 dilutions were used for molecular diagnosis.

Microarray design. The details of the design and selection process for the gene targets for RPM-Flu arrays were described in a previous publication (37). Briefly, after target genes were chosen based on the association between sequence diversity and clinically relevant phenotypic diversity, in silico modeling (21) was employed to select a set of probes that specifically recognize only the targeted clade of organisms while accounting for all or most of the recognized variants (i.e., strains or subtypes) within that clade. The RPM-Flu arrays (TessArray RPM-Flu 3.0 and 3.1; TessArae, LLC, Potomac Falls, VA) were designed to maximize coverage of respiratory pathogens, targeting all 16 *HA* and 9 *NA* alleles of avian influenza A viruses as well as three matrix (*M*) alleles, one nonstructural protein (*NS1*) allele, and one polymerase basic subunit 2 (*PB2*) region (see Table S1 in the supplemental material). About 30% of the RPM-Flu arrays are dedicated to the detection of influenza viruses. Because numerous respiratory pathogens needed to be detected using a limited amount of microarray space, most *HA* and *NA* genes were represented by a single sequence optimized for prevalent strains of influenza A viruses circulating in the avian population within the last few years (see Table S2 in the supplemental material). We have focused on ensuring the typing of the most common variants, while ensuring identification (with potentially less detailed typing results) for less common variants. There are instances where less common types will have more than 15% variation for some targets. This will not prevent detection of the influenza virus but may prevent subtyping to a single type and instead result in a list of several equally likely subtype identifications. Definite subtyping then relies on other targets on the array. The H10N7 samples discussed in Results serve as an example of this. The NA7 tile is designed based on the H7N7 subtype, and there is approximately 20% difference in the *NA* sequences of the H7N7 and the H10N7 subtypes. For a sample of H10N7, the sequence generated from the NA7 tile provides detection but indicates both H10N7 and H7N7 as being equally possible. The information from the HA10 (positive) and the HA7 (negative) subtypes allow a final determination that H10N7 has been detected. For each of the *HA*3, *HA*6, *HA*7, *NA*1, and *NA*8 genes, two different reference sequences were used because multiple sequence alignments revealed two major prevalent groups that could not be represented by a single reference sequence.

Primer design. Four independent multiplex primer mixes, separated to simplify primer design and multiplex optimization, were used to amplify a total of 187 targeted sequences represented on RPM-Flu arrays (298 primers). The gene-specific primer pairs for all targets on the RPM-Flu chips (see Table S1 in the supplemental material) were designed according to the criteria described previously (16, 17). To accommodate the genetic variation of RNA viruses and ensure amplification of closely related variants, a software script automated a primer selection algorithm developed by our group (unpublished data, available upon request) to select primers from defined primer regions of each target. Briefly, target sequences were used to search against GenBank to find all sequences sharing at least 80% sequence identity. Consensus sequences that covered the primer regions flanking the target sequences were generated from the sequences found. Primers were chosen to be between 18 and 25 bp in length, with predicted annealing temperatures between 55 and 60°C and at most one wobble nucleotide (M, R, S, W, Y, or K). When necessary, two primer pairs instead of one were used to amplify one target sequence.

Of the four multiplex primer mixes, one was dedicated to the influenza virus targets, including the aforementioned *HA* and *NA* alleles and the *M* genes from H3N2 and H1N1, which served as general markers of the presence of influenza A viruses. The *M*, *NS1*, and *PB2* genes from the H5N1 subtype were included to allow more-detailed analysis of critical regions subject to functional mutation (see Table S1 in the supplemental material).

Multiplex RT-PCR amplification. The multiplex RT-PCRs were performed as previously described (16), with the following modifications. For the RT step, primer LN was replaced by primer NLN (a random 9-mer with a linker sequence). One picogram each of two internal controls (NAC1 and triosephosphate isomerase) and 4 μl of the total nucleic acids extracted from either clinical specimens or laboratory controls were used. The RT reaction products were split into four 5-μl volumes for four different multiplex PCRs. Platinum *Taq* DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA) was replaced by

GoTaq DNA polymerase (Promega Corporation, Madison, WI) in the PCR. Linker primer NL instead of primer L was used with 50 to 150 nM each of primers from mixes 1 to 4 in the multiplex PCR. The amplification reaction was carried out with an initial incubation at 25°C for 10 min and then preliminary denaturation at 94°C for 2 min; followed by 16 cycles at 94°C for 30 s, 45 to 60°C for 30 s (with an incremental increase of 1°C per cycle), and 72°C for 90 s; and then 24 cycles at 94°C for 30 s and 60°C for 120 s. The amplified products from all four PCRs were combined into a single volume and subjected to purification and processing prior to hybridization to the RPM-Flu chips.

Microarray hybridization and analysis. The overall procedure of microarray processing protocol was based on a previously published procedure (see Fig. S1 in the supplemental material). Microarray hybridization and processing and image scanning and processing for production of FASTA output files were performed as previously described (16). Final pathogen identification was performed using Computer-Implemented Biological Sequence Identifier version 2.0 software (22), an automatic pathogen identification algorithm based on nucleic acid sequence alignment, which was developed and tested in detail in previous studies (16, 17).

Reference assays. Hemagglutination inhibition assays (HI) were performed at NAMRU3, following the World Health Organization Manual On Animal Influenza Diagnosis and Surveillance, version 2002.5, revision 1 (39). RT-PCR–ESI-MS was performed as previously described (30) at the Naval Health Research Center (NHRC; San Diego, CA). Additional assays, including conventional and/or real-time RT-PCR, were also performed as further confirmation at the NHRC and/or the Naval Research Laboratory (NRL; Washington, DC). Real-time RT-PCR was conducted to quantify the number of avian influenza virus genomes present in each sample (32). Furthermore, additional subtype-specific RT-PCR amplification assays were performed on discordant samples on the basis of both RPM-Flu and RT-PCR–ESI-MS results.

Full-length amplifications of *M* and *NS1* genes, using the universal primer set described by Hoffmann et al. (11), were also performed on discordant samples to obtain unbiased de novo sequence results. When the universal primer set did not produce sufficient material for conventional sequencing, subtype-specific RT-PCR amplifications were performed based on both RPM-Flu and RT-PCR–ESI-MS results. Amplified products from *HA*, *M*, *NA*, and *NS1* genes were purified and sent to Macrogen USA (Gaithersburg, MD) for Sanger/electrophoresis-based sequencing using either correspondent universal or specific primers.

Nucleotide sequence accession numbers. All nucleotide sequences reported in this study are available at GenBank (http://www.ncbi.nlm.nih.gov/GenBank/index.html) under accession numbers EU599273 to EU599315.

RESULTS

Analytical sensitivity. The limited representation of various *HA* and *NA* subtypes in available clinical samples constrained efforts to demonstrate the limits of detection for all *HA* and *NA* alleles. To validate the RPM platform for full-range influenza virus detection, artificially generated gene fragments (900 to 1,730 bp) (see Table S2 in the supplemental material) were used as an alternate means to estimate platform limits of detection. The analytical sensitivities for unequivocal detection and differentiation of all the *HA*1-16 and *NA*1-9 sequences represented on the RPM-Flu were between 10,000 and 1,000 genome copies per assay with various templates (data not shown). This sensitivity range with test templates was previously shown to correspond to excellent detection performance with clinical samples (17). These results indicated that the RPM-Flu approach should be an effective means of detecting and subtyping influenza virus directly from clinical samples.

Identification of influenza A virus isolates. Having demonstrated the capability of RPM-Flu for detecting and differentiating all *HA* and *NA* subtypes, a study was undertaken to characterize 37 cultured avian influenza virus specimens collected in the Middle East. This study effectively compared the utility of the RPM platform with respect to the traditional HI for avian influenza virus detection. The samples were additionally tested using alternate PCR methods available at the time (RT-PCR–ESI-MS and PCR assays, carried out at the NHRC). These samples were blinded with respect to all prior results and tested at the NRL by using the RPM-Flu platform. Sample identities were revealed only after the RPM results were finalized (Table 1).

For the 21 samples identified as the H5 serotype by HI, all methods produced identical results. Three additional samples, for which the HI results were not available, were H5N1 positive by PCR. Two samples identified as influenza A virus by HI were negative by all other methods, which suggested that sample degradation and/or low titers may have been involved. Of the remaining 11 samples, 6 samples were identified as other influenza virus serotypes and 5 were identified only as influenza A virus by HI. The PCR tests (specifically targeting influenza A virus, H1N1, H3N2, and H5N1) provided only species identification for these samples. Of the six samples for which HI made serotype identifications, RT-PCR–ESI-MS identified two samples as H5N1, three samples as H7N7, and one sample as influenza A virus, which contradicted the H7, H9, H10, and H11 identifications made by HI. RPM-Flu made the same serotype identifications as HI for five samples, and one sample was identified only as influenza A virus (Table 1). Of the five samples where HI offered only species identification, RT-PCR–ESI-MS (four samples) and RPM-Flu (three samples) offered serotype identification or identified them as influenza A virus. For the two samples where both methods offered serotype identifications, the results did not agree (Table 1).

Confirmation of influenza virus isolates by sequence analysis. Conventional sequencing of *HA*, *M*, *NA*, and *NS1* by use of universal primers (11) was attempted on the 11 discordant samples and 2 H5N1-positive samples as controls. When the universal amplification procedure failed, subtype-specific primers were employed based on the typing identifications made by both the RPM-Flu and the RT-PCR–ESI-MS platforms. In the event that conventional sequencing was successful, sequences generated for *M* and *NS1* genes did not provide conclusive information for subtype identification, but direct *HA* and *NA* gene sequencing agreed with the results from the RPM-Flu and HI platforms. In cases where HI failed to provide conclusive identification but the RPM-Flu provided detection, the identification was concordant with the de novo sequencing analysis (Table 1).

Identification of highly pathogenic influenza A strains. Translation of RPM-Flu resequencing data for the *HA* and *PB2* sequences of the 20 avian H5N1-positive samples confirmed that 13 samples contained the *HA* cleavage site and 15 samples indicated key amino acid substitutions in the PB2 protein. Eight samples had base calls before and after the location of the *NA* deletion. In these samples, this is a strong indication that a deletion is present. The remaining samples, unfortunately, had base calls only after the location of the *NA* deletion, so, while suggestive, it is possible that the absence of base calls before this location is associated with the low titer of the sample and not with the presence of a deletion. The overall results indicated that 75% of the H5N1 samples had at least one highly pathogenic strain marker and no conflicting indications. The remaining samples failed to provide base calls in all the marker locations; therefore, no clear determination of low-

TABLE 1. Identification of influenza A virus by RPM, HI, and RT-PCR–ESI-MS

Sample no.	Sample origin	Result[b] for:				
		HI (P1)	RPM	RT-PCR–ESI-MS	PCR	De novo sequencing
2006900207	Human	H5	H5N1	H5N1	H5N1	−
2006900588	Poultry[a]	H5	H5N1	H5N1	H5N1	−
2006900755	Human	H5	H5N1	H5N1	H5N1	−
2006900756	Human	H5	H5N1	H5N1	H5N1	−
2006900839	Chicken[a]	H5	H5N1	H5N1	H5N1	−
2006900840	Chicken[a]	H5	H5N1	H5N1	H5N1	−
2006900843	Chicken[a]	H5	H5N1	H5N1	H5N1	−
2006900844	Chicken[a]	H5	H5N1	H5N1	H5N1	−
2006900845	Chicken[a]	H5	H5N1	H5N1	H5N1	−
2006901162	Great egret	H5	H5N1	H5N1	H5N1	−
2006902782	Human	H5	H5N1	H5N1	H5N1	−
2006902786	Human	H5	H5N1	H5N1	H5N1	−
2006902834	Human	H5	H5N1	H5N1	H5N1	−
2006902838	Human	H5	H5N1	H5N1	H5N1	−
2006902991	Human	−	H5N1	H5N1	H5N?	−
2006902992	Human	−	H5N1	H5N1	H5N?	−
2006903458	Human	−	H5N1	H5N1	H5N1	−
2006906089	Poultry[a]	H5	H5N1	H5N1	H5N?	−
2006906375	Chicken[a]	H5	H5N1	H5N1	H5N1	−
2006906608	Duck[a]	H5	H5N1	H5N1	H5N1	−
2005909464	Goose[a]	H5	H5N1	H5N1	H5N1	−
2005909467	Duck[a]	H5	H5N1	H5N1	H5N1	−
2006900590	Poultry[a]	H5	H5N1	H5N1	H5N1	H?N1
2006902764	Chicken[a]	H5	H5N1	H5N1	H5N1	H5N1
2006905588	Goose	H7	H7N7	Flu A	H?N1	H7N7
2004909864	Shoveler	Flu A	H7N7	H9N2	Flu A	H7N?
2004900600	Shoveler	H10	H10N7	H7N7	Flu A	H10N7
2005912823	Teal	Flu A	H10N7	H7N7	Flu A	H10N7
2005912908	Teal	H10	H10N7	H7N7	Flu A	H10N7
2004900845	Shoveler	H10	H10N7 or H10N5	H5N1	Flu A	H10N7
2004900688	Teal	H11	H11N?	H7N7	Flu A	H11N?
2005912306	Yellow-legged bird	Flu A	H13N6 or H13N8	NEW	Flu A	H13N?
2005909888	Teal	Flu A	Flu A	H5N2	Flu A	−
2003920431	Teal	H9	Flu A	H5N1	H?N2	−
2005910801	Pintail	Flu A	Flu A	H5N3	Flu A	−
2005910876	Teal	Flu A	NEG	Low amp (H3N2)	NEG	−
2006902947	Human	Flu A	NEG	NEG	N1/NEG[c]	−

[a] Domestic.
[b] NEG, negative; Flu A, influenza A virus; Low amp, low-level amplification; −, no PCR results; P1, first passage after culture.
[c] PCR results provided by the NRL.

or high-pathogenicity strains could be made. The HA peptide translated from the two H7N7 samples indicated low virulence strains (data not shown).

Drug resistance information. In addition to tracking the mutations that were indicative of HPAI, RPM results provided the ability to track drug resistance markers. Previous work has shown that the NA protein may be characterized by four critical amino acid substitutions, E119V, R152K, H274Y, and R292K, associated with resistance against neuraminidase inhibitors. The amino acid substitution R292K was of particular significance, as this mutation results in complete resistance to oseltamivir and zanamavir (2). The results obtained from translation of the *NA* gene from RPM-Flu resequencing analysis of the H5N1-positive samples confirmed that 21 avian H5N1 samples with good base call rates harbored the E119V mutation. Furthermore, the R152K mutation was not observed in 17 out of 21 samples, the R292K mutation in 20 out of 21 samples, and the H274Y mutation in 9 out of 21 samples (data not shown). These data indicated that most of the avian H5N1 samples identified were presumably sensitive to neuraminidase inhibitors. Specific single-amino-acid substitutions in any one of four critical amino acids of the M2 protein, L26F, V27A/T, A30T/V, and S31N/R, have been associated with amantadine resistance (10, 29). Unfortunately, the results obtained from translation of the *M2* genes did not give conclusive results for amantadine resistance (data not shown).

DISCUSSION

The data presented herein demonstrate the capabilities of a resequencing microarray approach for influenza virus surveillance. The combination of bioinformatics (target design and pathogen identification algorithms) and amplification strategies applied in the RPM platform maximizes the potential for successful detection and facilitates molecular epidemiological surveillance by identifying and discriminating prevalent subtypes. The accuracy of the resequencing microarrays demonstrates utility not only for detection and identification of emerging and novel subtypes of avian influenza viruses but also for tracking of pathogenicity and drug resistance mutations. In

light of current concerns over the potential emergence and spread of highly pathogenic avian influenza viruses among humans leading to a pandemic, the RPM-Flu assay offers an ideal monitoring method, as it can detect all of the prevalent serotypes and resolve critical mutations. This assay potentially allows for specific and early intervention during outbreaks, thereby reducing the potential economic impact associated with persistent disease. Noteworthy is the fact that reassortment of various *HA* and *NA* alleles, which can readily expand avian influenza virus diversity and promote the emergence of epidemiologically important new strains, can be detected readily by the RPM platform since both of these genes are directly analyzed. This diagnostic approach is very effective for broad-spectrum, high-resolution surveillance situations. The higher per-assay costs of the RPM are offset by the fact that other methods would involve multiple separate tests requiring more time, materials, and cost.

The use of two additional PCR-based methods illustrates that other assay approaches can provide similar detection of avian influenza virus but that additional tests are required for the number of pathogens tested for by RPM-Flu to be matched. While detections of the presence of avian influenza virus were equivalent, different results were obtained from the methods for serotyping 11 of the 37 samples. Specific PCR remains one of the most inexpensive molecular diagnostic methods, making it useful for identifying the presence of current circulating strains. However, due to its limited scope, it is not easily applied for identifying reassortments or new variants or for broad-spectrum serotyping of diverse samples, as is described here for the non-H5N1 avian influenza virus samples tested.

RT-PCR–ESI-MS requires a high initial capital investment but is operationally a cost-effective method if the high-throughout capabilities of the platform can be utilized. Furthermore, this approach is able to provide more-detailed information beyond mere identification, such as its capacity to track the evolution of circulating human influenza virus. The previously reported capability of this method for serotype identification is largely confirmed in this study (30). RT-PCR–ESI-MS inferentially determines the identity of influenza viruses through base composition analysis of PCR segments of polymerase (*PB1*, *PB2*, and *PA*), *M*, *NS1*, and nucleoprotein (*NP*) genes (30). This inferential method may lead to the discrepancies seen in samples representing less prevalent subtypes with limited sequencing results in the database. Although this system is capable of reporting additional information, the tests and analysis applied to the samples in this study only provide serotyping information.

While the RPM-Flu chips demonstrate a very broad, high-confidence detection and analysis capability, the technology can be improved upon. For example, the current arrays, designed to provide comprehensive coverage of primarily human bacterial and viral pathogens, may not effectively provide detailed mutation and pathogenicity information for all subtypes of influenza virus. This impediment reflects the limits of microarray real estate, which demand that compromises between the depth and scope of coverage must be made. The limitation will become less of a factor as the capacity and cost of resequencing microarrays improve. In addition, no conclusive results could be obtained from the critical amino acids associated with amantadine drug resistance in the M2 protein. This is likely due to the fact that several nucleotide polymorphisms occur within the same 18-base-pair region with respect to the sequence used on the microarray. More specifically, clustered single nucleotide polymorphisms have a higher probability of not being identified on a resequencing microarray (21). Therefore, only ambiguous calls are made within the region, and no positive evidence of the mutation is provided.

Future iterations of RPM design can alleviate detection of bases in clustered mutation hotspots by including additional probes. Another issue that can be overcome in future iterations by modifying design strategies and analysis algorithms is the current difficulty in confirming sequence deletions. Finally, it should be noted that the absolute analytical sensitivity of the human influenza virus primers is slightly higher than that of the avian influenza virus primers, suggesting that adjustments in the primers may improve base call rates by providing more amplicon. To maximize the benefits of using a resequencing microarray for avian samples, an array that detects many pathogens of concern in bird samples will provide more information and could be placed on a smaller, less costly microarray. Efforts are currently ongoing to develop such an array.

ACKNOWLEDGMENTS

We thank Brandy Johnson, Igor Medintz, and Thomas O'Shaughnessy for critically reviewing the manuscript. We also thank the Centers for Disease Control and Prevention for providing the influenza A virus H5N1 (Asian lineage)-positive control.

Funding for this research was provided in part by the Office of Naval Research via the NRL base program. Partial support for this project came from TessArae, LLC (Potomac Falls, VA), through a cooperative research and development agreement. There are two pending patent applications, one for an amplification method and one for an analysis algorithm, that are related to this article. Clark Tibbetts is chief technology officer of TessArae, LLC, which is a for-profit organization. None of the authors from the NHRC or NAMRU3 have a financial conflict of interest.

We applied the sequence-determines-credit approach for determining the sequence of authors (35).

The opinions and assertions contained herein are those of the authors and are not to be construed as those of the U.S. Navy or military service at large.

REFERENCES

1. **Banks, J., E. S. Speidel, E. Moore, L. Plowright, A. Piccirillo, I. Capua, P. Cordioli, A. Fioretti, and D. J. Alexander.** 2001. Changes in the haemagglutinin and the neuraminidase genes prior to the emergence of highly pathogenic H7N1 avian influenza viruses in Italy. Arch. Virol. **146:**963–973.
2. **Behrens, G., R. Gottschalk, L. Gürtle, T. C. Harder, S. Korsman, G. Reyes-Terán, M. Stoll, O. Werner, and G. van Zyl.** 2006. Influenza Report 2006. Flying Publisher, Wuppertal, Germany. http://www.influenzareport.com/influenzareport2006.pdf.
3. **Bright, R. A., M. J. Medina, X. Xu, G. Perez-Oronoz, T. R. Wallis, X. M. Davis, L. Povinelli, N. J. Cox, and A. I. Klimov.** 2005. Incidence of adamantane resistance among influenza A (H3N2) viruses isolated worldwide from 1994 to 2005: a cause for concern. Lancet **366:**1175–1181.
4. **Bright, R. A., D. K. Shay, B. Shu, N. J. Cox, and A. I. Klimov.** 2006. Adamantane resistance among influenza A viruses isolated early during the 2005–2006 influenza season in the United States. JAMA **295:**891–894.
5. **Brown, I. H.** 2006. Advances in molecular diagnostics for avian influenza. Dev. Biol. (Basel) **124:**93–97.
6. **Davignon, L., E. A. Walter, K. M. Mueller, C. P. Barrozo, D. A. Stenger, and B. Lin.** 2005. Use of resequencing oligonucleotide microarrays for identification of *Streptococcus pyogenes* and associated antibiotic resistance determinants. J. Clin. Microbiol. **43:**5690–5695.
7. **Ellis, J. S., J. W. Smith, S. Braham, M. Lock, K. Barlow, and M. C. Zambon.** 2007. Design and validation of an H5 TaqMan real-time one-step reverse transcription-PCR and confirmatory assays for diagnosis and verification of influenza A virus H5 infections in humans. J. Clin. Microbiol. **45:**1535–1543.
8. **Fan, J., K. J. Henrickson, and L. L. Savatski.** 1998. Rapid simultaneous diagnosis of infections with respiratory syncytial viruses A and B, influenza

viruses A and B, and human parainfluenza virus types 1, 2, and 3 by multiplex quantitative reverse transcription-polymerase chain reaction-enzyme hybridization assay (Hexaplex). Clin. Infect. Dis. **26:**1397–1402.
9. **Fouchier, R. A., V. Munster, A. Wallensten, T. M. Bestebroer, S. Herfst, D. Smith, G. F. Rimmelzwaan, B. Olsen, and A. D. Osterhaus.** 2005. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. J. Virol. **79:**2814–2822.
10. **Hay, A. J., A. J. Wolstenholme, J. J. Skehel, and M. H. Smith.** 1985. The molecular basis of the specific anti-influenza action of amantadine. EMBO J. **4:**3021–3024.
11. **Hoffmann, E., J. Stech, Y. Guan, R. G. Webster, and D. R. Perez.** 2001. Universal primer set for the full-length amplification of all influenza A viruses. Arch. Virol. **146:**2275–2289.
12. **Horimoto, T., and Y. Kawaoka.** 1995. Direct reverse transcriptase PCR to determine virulence potential of influenza A viruses in birds. J. Clin. Microbiol. **33:**748–751.
13. **Kessler, N., O. Ferraris, K. Palmer, W. Marsh, and A. Steel.** 2004. Use of the DNA flow-thru chip, a three-dimensional biochip, for typing and subtyping of influenza viruses. J. Clin. Microbiol. **42:**2173–2185.
14. **Lee, W. M., K. Grindle, T. Pappas, D. J. Marshall, M. J. Moser, E. L. Beaty, P. A. Shult, J. R. Prudent, and J. E. Gern.** 2007. High-throughput, sensitive, and accurate multiplex PCR-microsphere flow cytometry system for large-scale comprehensive detection of respiratory viruses. J. Clin. Microbiol. **45:**2626–2634.
15. **Li, J., S. Chen, and D. H. Evans.** 2001. Typing and subtyping influenza virus using DNA microarrays and multiplex reverse transcriptase PCR. J. Clin. Microbiol. **39:**696–704.
16. **Lin, B., K. M. Blaney, A. P. Malanoski, A. G. Ligler, J. M. Schnur, D. Metzgar, K. L. Russell, and D. A. Stenger.** 2007. Using a resequencing microarray as a multiple respiratory pathogen detection assay. J. Clin. Microbiol. **45:**443–452.
17. **Lin, B., A. P. Malanoski, Z. Wang, K. M. Blaney, A. G. Ligler, R. K. Rowley, E. H. Hanson, E. von Rosenvinge, F. S. Ligler, A. W. Kusterbeck, D. Metzgar, C. P. Barrozo, K. L. Russell, C. Tibbetts, J. M. Schnur, and D. A. Stenger.** 2007. Application of broad-spectrum, sequence-based pathogen identification in an urban population. PLoS ONE **2:**e419.
18. **Lin, B., A. P. Malanoski, Z. Wang, K. M. Blaney, A. G. Ligler, R. K. Rowley, E. H. Hanson, E. von Rosenvinge, F. S. Ligler, A. W. Kusterbeck, D. Metzgar, C. P. Barrozo, K. L. Russell, C. Tibbetts, J. M. Schnur, and D. A. Stenger.** 2007. Application of broad-spectrum, sequence-based pathogen identification in an urban population. PLoS ONE **2:**e419.
19. **Lin, B., Z. Wang, G. J. Vora, J. A. Thornton, J. M. Schnur, D. C. Thach, K. M. Blaney, A. G. Ligler, A. P. Malanoski, J. Santiago, E. A. Walter, B. K. Agan, D. Metzgar, D. Seto, L. T. Daum, R. Kruzelock, R. K. Rowley, E. H. Hanson, C. Tibbetts, and D. A. Stenger.** 2006. Broad-spectrum respiratory tract pathogen identification using resequencing DNA microarrays. Genome Res. **16:**527–535.
20. **Mahony, J., S. Chong, F. Merante, S. Yaghoubian, T. Sinha, C. Lisle, and R. Janeczko.** 2007. Development of a respiratory virus panel test for detection of twenty human respiratory viruses by use of multiplex PCR and a fluid microbead-based assay. J. Clin. Microbiol. **45:**2965–2970.
21. **Malanoski, A. P., B. Lin, and D. A. Stenger.** 2008. A model of base-call resolution on broad-spectrum pathogen detection resequencing DNA microarrays. Nucleic Acids Res. **36:**3194–3201.
22. **Malanoski, A. P., B. Lin, Z. Wang, J. M. Schnur, and D. A. Stenger.** 2006. Automated identification of multiple micro-organisms from resequencing DNA microarrays. Nucleic Acids Res. **34:**5300–5311.
23. **Munch, M., L. P. Nielsen, K. J. Handberg, and P. H. Jorgensen.** 2001. Detection and subtyping (H5 and H7) of avian type A influenza virus by reverse transcription-PCR and PCR-ELISA. Arch. Virol. **146:**87–97.
24. **Munster, V. J., C. Baas, P. Lexmond, J. Waldenstrom, A. Wallensten, T. Fransson, G. F. Rimmelzwaan, W. E. Beyer, M. Schutten, B. Olsen, A. D. Osterhaus, and R. A. Fouchier.** 2007. Spatial, temporal, and species variation in prevalence of influenza A viruses in wild migratory birds. PLoS Pathog. **3:**e61.
25. **Olsen, B., V. J. Munster, A. Wallensten, J. Waldenstrom, A. D. Osterhaus, and R. A. Fouchier.** 2006. Global patterns of influenza a virus in wild birds. Science **312:**384–388.
26. **Pejcic, B., R. De Marco, and G. Parkinson.** 2006. The role of biosensors in the detection of emerging infectious diseases. Analyst **131:**1079–1090.
27. **Pickering, J. W., T. B. Martins, M. C. Schroder, and H. R. Hill.** 2002. Comparison of a multiplex flow cytometric assay with enzyme-linked immunosorbent assay for auantitation of antibodies to tetanus, diphtheria, and *Haemophilus influenzae* type b. Clin. Diagn. Lab. Immunol. **9:**872–876.
28. **Pourmand, N., L. Diamond, R. Garten, J. P. Erickson, J. Kumm, R. O. Donis, and R. W. Davis.** 2006. Rapid and highly informative diagnostic assay for H5N1 influenza viruses. PLoS ONE **1:**e95.
29. **Puthavathana, P., P. Auewarakul, P. C. Charoenying, K. Sangsiriwut, P. Pooruk, K. Boonnak, R. Khanyok, P. Thawachsupa, R. Kijphati, and P. Sawanpanyalert.** 2005. Molecular characterization of the complete genome of human influenza H5N1 virus isolates from Thailand. J. Gen. Virol. **86:**423–433.
30. **Sampath, R., K. L. Russell, C. Massire, M. W. Eshoo, V. Harpin, L. B. Blyn, R. Melton, C. Ivy, T. Pennella, F. Li, H. Levene, T. A. Hall, B. Libby, N. Fan, D. J. Walcott, R. Ranken, M. Pear, A. Schink, J. Gutierrez, J. Drader, D. Moore, D. Metzgar, L. Addington, R. Rothman, C. A. Gaydos, S. Yang, K. St. George, M. E. Fuschino, A. B. Dean, D. E. Stallknecht, G. Goekjian, S. Yingst, M. Monteville, M. D. Saad, C. A. Whitehouse, C. Baldwin, K. H. Rudnick, S. A. Hofstadler, S. M. Lemon, and D. J. Ecker.** 2007. Global surveillance of emerging influenza virus genotypes by mass spectrometry. PLoS ONE **2:**e489.
31. **Sengupta, S., K. Onodera, A. Lai, and U. Melcher.** 2003. Molecular detection and identification of influenza viruses by oligonucleotide microarray hybridization. J. Clin. Microbiol. **41:**4542–4550.
32. **Stone, B., J. Burrows, S. Schepetiuk, G. Higgins, A. Hampson, R. Shaw, and T. Kok.** 2004. Rapid detection and simultaneous subtype differentiation of influenza A viruses by real time PCR. J. Virol. Methods **117:**103–112.
33. **Templeton, K. E., S. A. Scheltinga, M. F. Beersma, A. C. Kroes, and E. C. Claas.** 2004. Rapid and sensitive method using multiplex real-time PCR for diagnosis of infections by influenza A and influenza B viruses, respiratory syncytial virus, and parainfluenza viruses 1, 2, 3, and 4. J. Clin. Microbiol. **42:**1564–1569.
34. **Townsend, M. B., E. D. Dawson, M. Mehlmann, J. A. Smagala, D. M. Dankbar, C. L. Moore, C. B. Smith, N. J. Cox, R. D. Kuchta, and K. L. Rowlen.** 2006. Experimental evaluation of the FluChip diagnostic microarray for influenza virus surveillance. J. Clin. Microbiol. **44:**2863–2871.
35. **Tscharntke, T., M. E. Hochberg, T. A. Rand, V. H. Resh, and J. Krauss.** 2007. Author sequence and credit for contributions in multiauthored publications. PLoS Biol. **5:**e18.
36. **Wang, Z., L. T. Daum, G. J. Vora, D. Metzgar, E. A. Walter, L. C. Canas, A. P. Malanoski, B. Lin, and D. A. Stenger.** 2006. Identifying influenza viruses with resequencing microarrays. Emerg. Infect. Dis. **12:**638–646.
37. **Wang, Z., A. P. Malanoski, B. Lin, C. Kidd, N. C. Long, K. M. Blaney, D. C. Thach, C. Tibbetts, and D. A. Stenger.** 2008. Resequencing microarray probe design for typing genetically diverse viruses: human rhinoviruses and enteroviruses. BMC Genomics **9:**577.
38. **Whiley, D. M., and T. P. Sloots.** 2005. A 5′-nuclease real-time reverse transcriptase-polymerase chain reaction assay for the detection of a broad range of influenza A subtypes, including H5N1. Diagn. Microbiol. Infect. Dis. **53:**335–337.
39. **World Health Organization.** 2002. WHO manual on animal influenza diagnosis and surveillance. World Health Organization, Geneva, Switzerland.
40. **Wright, P. F., and R. G. Webster.** 2001. Orthomyxoviruses, p. 1533–1579. *In* D. M. Knipe and P. M. Howley (ed.), Fields virology, 4th ed., vol. 1. Lippincott Williams & Wilkins, Philadelphia, PA.